J Mater Sci: Mater Med (2011) 22:2065–2070
DOI 10.1007/s10856-011-4382-8

In vitro biocompatibility of modified potassium fluorrichterite and potassium fluorrichterite-fluorapatite glass–ceramics

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Received: 21 September 2009 / Accepted: 15 June 2011 / Published online: 25 June 2011

Abstract Potassium fluorrichterite ($KNaCaMg_5Si_8O_{22}F_2$) glass–ceramics were modified by either increasing the concentration of calcium in the glass (GC5), or by the addition of P_2O_5 to produce potassium fluorrichterite-fluorapatite (GP2). The solubility of the stoichiometric composition (GST), GC5 and GP2 were measured using the standard test described in ISO 6872:1995 (Dental Ceramics). Ion release profiles were determined for Si, Ca, Mg, Na, K and P using inductively coupled plasma mass spectrometry and fluoride ion (F^-) concentration was measured using an ion-selective electrode. The cytotoxicity of all compositions was assessed using cultured rat osteosarcoma cells (ROS, 17/2.8). Cell response was qualitatively assessed using scanning electron microscopy (SEM) and quantitatively using the Alamar blue assay. GST was the least soluble and also released the lowest concentration of ions following immersion in water. Of the modified compositions, GC5 demonstrated intermediate solubility but the greatest ion release while GP2 exhibited the highest solubility. This was most likely due to GC5 having the greatest proportion of residual glass following crystallisation. The mass loss exhibited by GP2 may have been due in part to the partial disintegration of the surface of specimens during solubility testing. SEM demonstrated that all compositions supported the growth of healthy ROS cells on their surfaces, and this data was further supported by the quantitative Alamar blue assay.

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1 Introduction

Bioceramics are used widely in bone tissue reconstruction and augmentation, although, the most common materials are relatively brittle and unsuitable for load-bearing applications. Calcium phosphates such as hydroxyapatite (HA) remain the most popular osteoconductive bone substitute materials, but their poor mechanical properties limit their use to particulate fillers, graft expanders and implant coatings [1]. Bioactive glasses, such as 45S5 bioglass, have the ability to encourage the formation of a HA layer in vitro on their surface and exhibit good bone bonding properties in vivo [2]. However, the mechanical properties of all bioactive glasses are also inadequate for load-bearing applications. Attempts have been made to address the poor mechanical properties by instead developing relatively tough glass–ceramics. Apatite-wollastonite (AW) glass–ceramic has proved to be the most successful to date, with good mechanical strength, toughness and stability in the biological environment [3, 4]. However, manufacturing AW devices requires a complex procedure based on powder processing as it is unsuitable for casting due to surface nucleation. There is therefore a demand for a tough, osteoconductive glass–ceramic that could more easily be processed to form complex or custom shapes for use as a bone tissue substitute in reconstructive and plastic surgery.

Chain silicate glass–ceramics have the necessary mechanical properties, but unmodified compositions are not osteoconductive and have been reported to elicit a poor tissue response after implantation [5]. Attempts to reduce solubility

by modifying the relative concentrations of Na and K in the composition have been similarly disappointing in terms of bone tissue response [6]. While adjusting monovalent cations (Na and K) was not effective, increasing the relative concentration of CaO and/or the addition of P_2O_5 to crystallize apatite has been reported to improve the ostoconductive potential of fluorocanasite glass–ceramics in vitro [7] and in vivo [8], while retaining excellent mechanical properties [9]. Within the chain silicate family of glass ceramics, potassium fluorrichterite ($KNaCaMg_5Si_8O_{22}F_2$) compositions are also promising candidates as bone substitute materials. The mechanical properties [10, 11] and phase evolution [12] of compositions modified with the intention of improving bone tissue response has been the subject of detailed study. They have high flexural strengths ($\sim$250 MPa) and fracture toughness ($\sim$2.7 $MPam^{1/2}$) [11, 13, 14], bulk nucleate, and have relatively low liquidus temperature which permits casting to net shape [11, 12]. X-ray diffraction (XRD) and transmission electron microscopy (TEM) studies have confirmed that GST is completely crystalline, with potassium fluorrichterite being the only crystalline phase detected [11]. GC5 has one additional crystalline phase (Diopside) while GP2 also contains fluorapatite, enstatite, tridymite and sodium potassium magnesium silicate [11, 15]. The residual glass phase in these compositions was estimated by TEM to be between 0 and 5% by volume [11].

While detailed knowledge of the phase evolution and mechanical properties of these modified glass–ceramics has been published, little is known regarding their biocompatibility. Recent research has shown that the modified compositions reported here are capable of forming an apatite layer on their surfaces following immersion in simulated body fluid [16]. Prior to selection of compositions for in vivo testing, however, it is necessary to investigate other aspects of in vitro biocompatibility. The aim of this study was to therefore investigate the solubility, ion release and in vitro biocompatibility of modified potassium fluorrichterite and potassium fluorrichterite-fluorapatite glass–ceramics using cultured bone cells.

2 Materials and methods

2.1 Glass preparation

Three glass–ceramic compositions based on potassium fluorrichterite ($KNaCaMg_5Si_8O_{22}F_2$) (KFR), were evaluated in this study; stoichiometric (GST), GST with 5 mol% CaO substituted for MgO (GC5) and GST with 2 mol% P_2O_5 added (GP2). These compositions were originally developed by Mirsaneh et al. [10] as members within extended series that investigated the glass formability, microstructure and mechanical properties of KFR based glass ceramics. The glass batches were prepared using silica (Loch Aline sand 99.5% SiO_2) and reagent grade chemicals (Fisher Scientific and Sigma-Aldrich). The chemical compositions in mol% are listed in Table 1. The glasses were melted in uncovered platinum (with 2% rhodium) crucibles at 1400°C for 3 h in an electric furnace, and stirred for the final 2 h of the melt with a platinum stirrer at 60 rpm. All compositions were cast as glass onto a pre-heated steel plate, annealed in a muffle furnace between 520–580°C and cooled to room temperature at 1°C/min. As-cast glass was core drilled (Sealey Power Products, Sealey Group, Bury St. Edmunds, UK) using a diamond core drill (HABIT core drill, 12 mm internal diameter, 1 mm thickness, Abrasive Technology Ltd, London, UK) to produce glass cylinders, which were cut using a diamond blade (Diamond wafering blade 15 HC, Buehler, USA) into discs. To crystallise the specimen, the discs were heat treated for 4 h in a muffle furnace (Eurotherm 818 Programmable Furnace, Lenton Thermal Design, Hope Valley, Derbyshire, UK) rising at 5°C/min to 950°C for GST and 1000°C for GC5 and GP2 and cooled at 5°C/min to room temperature.

2.2 Solubility testing

Chemical solubility was determined using ISO 6872:1995 (Dental Ceramic Standard) [17]. Ten discs per composition were fabricated to the required size (12 × 1.6 mm) using silicon carbide grinding paper (Buehler-Met II, Buehler UK Ltd, Coventry, UK) to a P600 grade finish, rinsed thoroughly with distilled water, placed in a glass petri dish and dried at 160 ± 5°C in a thermostatically controlled oven for 4 h. Following which, they were cooled and individually weighed to the nearest 0.1 mg on an electronic balance (Mettler AJ100, Mettler Toledo Ltd, Leicester, UK). The samples were handled with nylon tweezers at all times to minimize contamination and damage to the specimen. The samples were placed in the extraction apparatus and were refluxed with 4% acetic acid solution at the reflux rate of approximately 3 cycles per hour, for 16 h, at 80°C. Following extraction, the samples were rinsed with distilled water, dried and weighed to the closest 0.1 mg. The average mass difference divided by the surface area of each disc gave the solubility value in $\mu g/cm^2$. The specimens were analysed before and after testing, using scanning electron microscopy (SEM) (CamScan II, Obducat CamScan Ltd, Cambridgeshire, UK).

2.3 Ion release

Discs ($n = 4$, 12 × 2 mm) of each composition were immersed in 25 ml plastic cylindrical containers with a conical base, filled with 20 ml of ultrapure water

Table 1 Composition in mol% of the test compositions

CODE		SiO_2	Na_2O	K_2O	MgO	CaF_2	CaO	P_2O_5
GST	Stoichiometric	53.37	3.33	3.33	33.35	6.62	–	–
GC5	5 mol% CaO subs. for MgO in GST	53.37	3.33	3.33	28.35	6.62	5	–
GP2	2 mol% P_2O_5 added to GST	52.26	3.26	3.26	32.66	6.56	–	2

Table 2 Solubility measured using ISO 6872:1995

Composition	Solubility μg/cm^2
GST	61.2
GC5	420.4
GP2	1232.7

(Ultrapure water purification system, Millipore, Watford, England) and sealed with a plastic screw top. Containers containing only ultrapure water were used as control. All specimen containers were placed in a water bath at 37°C and readings were taken after 1, 7 and 28 days. Fluoride ion release assessment was carried out using an ion-selective electrode (Orion Research Inc, Cambridge, USA) after the addition of TISAB II (Total Ionic Strength Adjustment Buffer, Sigma-Aldrich, UK), which was used as a decomplexing solution. The probe was calibrated between 0.1 and 1 ppm or between 1 and 10 ppm, as required, using the manufacturers standard solutions. The concentration of the other ions (P, Si, Mg, Ca, Al, Na & K) were measured at the same time points using inductively coupled plasma-mass spectroscopy (ICP-MS).

2.4 Cell culture

Discs ($n = 4$, 12 × 2 mm) of each composition were tested along with 4 slip-cast hydroxyapatite discs (Ceramisys, Sheffield, UK) used as reference materials. After thoroughly washing with distilled water and phosphate buffered saline (PBS), the samples were autoclaved (121°C for 15 min at 15 psi pressure). Rat osteosarcoma cells (ROS 17/2.8, Merck) were used as these have been shown to be good indicators for biocompatibility [18–20]. The cells were seeded in a 24 well plate containing the test specimen and control samples. The seeding density was 1.25×10^4 cells/ml with a total volume within each well of 1 ml. A non-material control (cells only) was included for comparison. After 72 h, the cellular response to the test materials was assessed qualitatively (cell morphology, adaptation, etc.) by SEM and quantitatively (measurement of cellular respiratory rate) by Alamar Blue assay. Alamar Blue (AB) is a water-soluble dye that has been used for quantifying in vitro viability of various cells [21]. As it is extremely stable, non-destructive and non toxic to the cells it is considered superior to classical tests for cell viability such as the MTT assay [17].

3 Results

3.1 Solubility

Solubility data for GST, GC5 and GP2 determined using the ISO 6872:1995 standard test is given in Table 2. ISO 6872 is a test developed for dental ceramics but it may also be used to predict dissolution in vivo. The stoichiometric composition (GST) showed a low solubility (61.2 μg/cm^2) and SEM images taken post-solubility testing (Fig. 1b) showed a largely intact surface with none of the morphological characteristics associated with surface dissolution. There was, however, evidence of damage to the edges of some specimens (Fig. 1c). Similar features have been reported previously in the literature and are considered to be a limitation of the current ISO test [22]. SEM images of GC5 showed evidence of surface dissolution, which was consistent with acidic degradation (Fig. 2). GP2, however, showed a pattern of surface dissolution that appeared to show shedding of particulate debris rather than dissolution at the surface (Fig. 3).

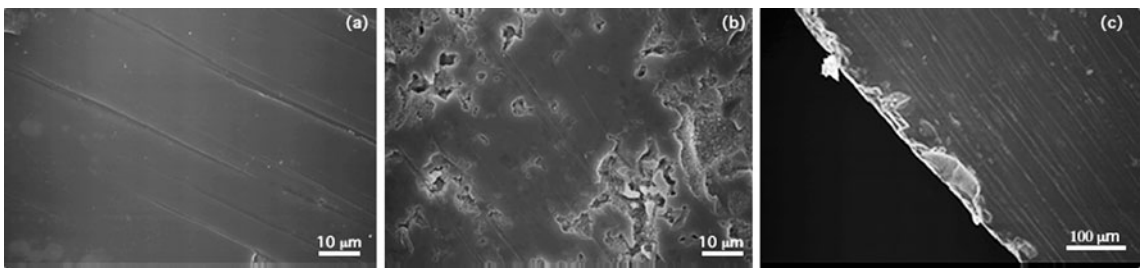

Fig. 1 SEM micrographs showing (**a**) the surface of GST before solubility testing (**b**) following solubility testing and (**c**) showing 'edge effects'

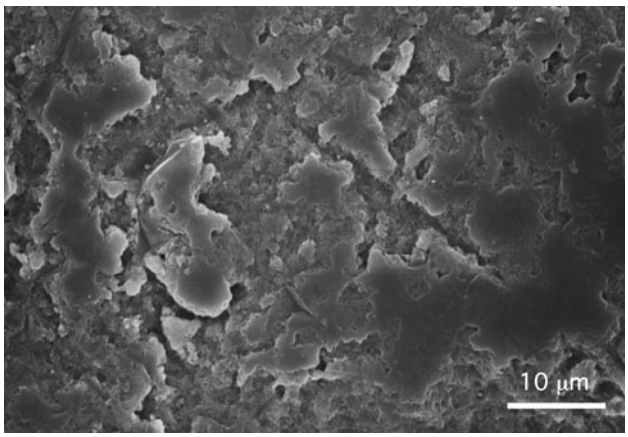

Fig. 2 SEM micrograph showing the surface of GC5 after solubility testing

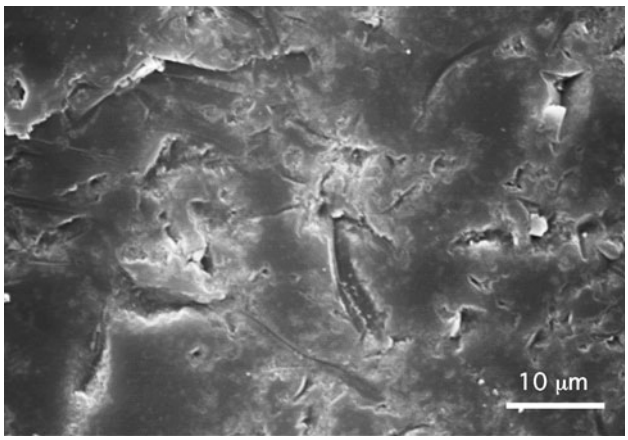

Fig. 3 SEM micrograph showing the surface of GP2 after solubility testing

3.2 Ion release

Ion release was determined over a period of approximately 1 month with measurements taken after 1, 7 and 28 days (Table 3). Background measurements were subtracted and all values were converted to μ mol/mm^2 to facilitate comparison. In agreement with its low mass loss, the total ion release from GST was also low with only Si (20.69 ± 3.27 μ mol/mm^2) and Mg (12.92 ± 1.85 μ mol/mm^2) detected after 28 days. The ion release from GP2 was greater than GST as expected due to its higher solubility. Si (132.03 ± 28.6 μ mol/mm^2) and Mg (41.85 ± 8.96 μ mol/mm^2) were again present at the highest concentrations, and P (19.22 ± 7.97 μ mol/mm^2) was also detected. The ion release profile from GC5 showed concentrations that were significantly greater than those seen for GST or GP2. Si (371.85 ± 156.35 μ mol/mm^2) was the highest concentration ion detected after 28 days with significant proportions of K (162.33 ± 98.09 μ mol/mm^2), Na (104.56 ± 64.21 μ mol/mm^2), and Ca (74.63 ± 32.1 μ mol/mm^2) also observed.

3.3 Cell culture

After 72 h, all glass ceramics showed ROS cells in close apposition to the surface (shown for GST in Fig. 4) and similar in appearance as those detected on different

Table 3 Ionic release profiles of GST, GP2 and GC5 over a period of 28 days (all values in μ mol/mm^2)

Composition	Ion	Day 1 (all values in μ mol/mm^2)	Day 7	Day 28
GST	P	0	0	0.07 ± 0.1
	Ca	0.31 ± 0.05	1.17 ± 0.2	2.8 ± 0.46
	K	0.83 ± 0.16	1.56 ± 0.21	3.81 ± 2.03
	Si	4.57 ± 0.94	9.65 ± 1.74	20.69 ± 3.27
	Mg	2.01 ± 0.42	5.28 ± 0.84	12.92 ± 1.85
	Na	0.62 ± 0.09	1.42 ± 0.27	2.75 ± 0.71
GC5	P	0.07 ± 0.07	0.07 ± 0.13	0.07 ± 0.1
	Ca	3.56 ± 1.88	13.48 ± 5.49	74.63 ± 32.1
	K	6.17 ± 4.64	32.54 ± 18.51	162.33 ± 98.09
	Si	29.03 ± 20.69	105.97 ± 57.7	371.85 ± 156.35
	Mg	1.34 ± 0.25	4.19 ± 0.42	15.94 ± 3.77
	Na	4.35 ± 3.02	21.28 ± 11.97	104.56 ± 64.21
GP2	P	0.4 ± 0.2	2.24 ± 1.05	19.22 ± 7.97
	Ca	0.56 ± 0.15	2.09 ± 0.61	5.49 ± 1.78
	K	1.72 ± 0.26	4.43 ± 0.89	24.46 ± 8.66
	Si	40.57 ± 5.88	60.75 ± 9.65	132.03 ± 28.6
	Mg	3.35 ± 0.59	12.58 ± 2.43	41.85 ± 8.97
	Na	0.89 ± 0.27	2.22 ± 0.53	10.91 ± 3.55

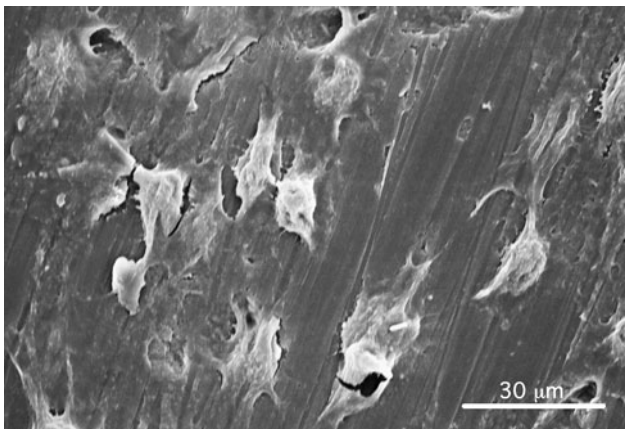

Fig. 4 Secondary electron SEM micrograph of ROS cells cultured for 72 h on GST showing osteoblast-like cells in close apposition

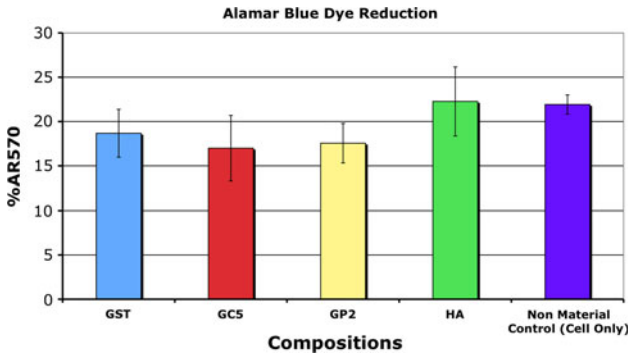

Fig. 5 Alamar blue dye reduction assay after 72 h for GST, GC5, GP2, HA and the non-material control (cell-only)

biomaterials [18–20]. The number of cells, cellular morphology and adaptation appeared similar for all test compositions. The Alamar blue assay performed in fresh media following 72 h culture indicated that all test materials were similarly biocompatible in vitro (Fig. 5) with similar levels of respiratory activity suggesting similar cell numbers ($P < 0.05$) albeit lower than HA and the tissue culture polystyrene reference wells (cell-only).

4 Discussion

The low degree of solubility demonstrated by GST is most likely due to the highly crystalline microstructure with no detectable residual glass [12], as the amount of residual glass in a glass ceramic is generally proportional to its solubility [22]. No published value exists for the level of solubility that a glass ceramic should exhibit in order to be viable as a bone substitute. When compared with the values for dental ceramics published in the ISO standard [17], GST exhibited solubility that would be equivalent of a material suitable for use as a veneering ceramic for dental crowns (<100 µg/cm^2), while the solubility exhibited by GC5 and GP2 would make them suitable as core ceramics for dental crowns (<2000 µg/cm^2). Given the strong association of high solubility with poor biocompatibility in many previous studies of glass ceramics, it seems reasonable to suggest that these less soluble systems will perform better in vivo [5, 6, 8].

Mass loss data for GP2 was influenced by the disaggregation of specimens under acidic test conditions with the generation of particulate debris. This accounts for the lack of correlation between mass loss and ion release, which is a better indicator of the solubility of these glasses. This was illustrated by SEM that showed evidence of surface flaking during the solubility tests. GC5, which was designed to have the greatest proportion of residual glass [11], as predicted, showed the greatest ion release, consistent with similar chain silicate glass ceramics.

Though, there were no demonstrable differences in cell culture or respiratory activity between the test compositions, neither was there evidence of cytotoxicity in any of the materials tested. While the data here suggests that modifying fluorrichterite compositions does not dramatically alter in vitro biocompatibility, it is likely that differences will be identified in vivo. Freeman et al. demonstrated that the controlled crystallization of apatite in an otherwise 'bioinert' glass composition was able to impact bone bonding or osteoconductivity [23]. Therefore, we believe that solubility of the residual glass phase and introduction of a crystalline apatite phase are capable of independently influencing both biocompatibility and osteoconductivity. Further in vivo studies are therefore required to test this hypothesis.

5 Conclusions

GST and GC5 exhibited solubility and ion release profiles that are consistent with similar chain silicate glass ceramic materials. During solubility testing, these compositions showed evidence of surface dissolution and some loss of particulate debris from the edges. GP2 however, was prone to flaking and loss of particulate matter over its entire surface when subjected to standard accelerated solubility testing. As a consequence, the mass lost appeared substantially greater than could be accounted for by ion release. Overall, GST exhibited the lowest solubility (61 µg/cm^2) and ion release due to its high degree of crystallinity. All elemental species present in the glass ceramics were detected in solution, with Si at the highest relative concentration. Experimental compositions appeared to show good in vitro biocompatibility as they supported the growth of cultured ROS 17/2.8 cells on their surfaces.

Acknowledgments The authors would like to express their gratitude to Prof. John Nicholson and Dr. Kunle Awosanya, University of Greenwich for their assistance with ICP-MS. They also acknowledge Merck Inc. for their permission to use the ROS 17/2.8 cell line in in vitro biocompatibility studies.

References

1. Brook IM, Lamb DJ. Correction of local alveolar defects by implantation of hydroxyapatite: a preliminary study. Br Dent J. 1986;161(2):68–70.
2. Hench LL. The story of bioglass. J Mater Sci Mater Med. 2006; 17(11):967–78.
3. Yoshii S, et al. Strength of bonding between A-W glass–ceramic and the surface of bone cortex. J Biomed Mater Res. 1988;22(3 Suppl):327–38.
4. Kokubo T, et al. Apatite and wollastonite containing glass-ceramics for prosthetic applications. Bull Inst Chem Res Kyoto Univ. 1982;60:260–8.
5. da Rocha Barros VM, et al. In vivo bone tissue response to a canasite glass–ceramic. Biomaterials. 2002;23:2895–9000.
6. da Rocha Barros VM, Liporaci JL Jr, Rosa AL, Junqueira MC, de Oliveira PT, Johnson A, van Noort R. Bone response to three different chemical compositions of fluorocanasite glass-ceramic. J Biomed Mater Res A. 2007;83(2):480–3.
7. Miller CA, et al. Formation of apatite layers on modified canasite glass–ceramics in simulated body fluid. J Biomed Mater Res. 2002;59(3):473–80.
8. Bandyopadhyay-Ghosh SFP, Johnson A, Felipucci DN, Reaney IM, Salata LA, Brook IM, Hatton PV. Osteoconductivity of modified fluorocanasite glass–ceramics for bone tissue augmentation and repair. J Biomed Mater Res A. 2010;94(3):760–8.
9. Denry IL, Holloway JA. Effect of crystallization heat treatment on the microstructure and biaxial strength of fluorrichterite glass–ceramics. J Biomed Mater Res B. 2007;80(2):454–9.
10. Denry IL, Holloway JA. Elastic constants, Vickers hardness, and fracture toughness of fluorrichterite-based glass–ceramics. Dent Mater. 2004;20(3):213–9.
11. Mirsaneh M, et al. Characterization of high-fracture toughness K-fluorrichterite–fluorapatite glass–ceramics. J Amer Ceram Soc. 2004;87(2):240–6.
12. Mirsaneh M, et al. Effect of CaF2 and CaO substituted for MgO on the phase evolution and mechanical properties of K-fluorrichterite glass–ceramics. J Amer Ceram Soc. 2006;89(2): 587–95.
13. Beall GH and Meagles JE Jr. Potassium fluorrichterite glass ceramics and method U.S. Patent, Editor. 1984: United States.
14. Beall GH. Chain silicate glass–ceramics. J Non-Cryst Solids. 1991;129:163–73.
15. Mirsaneh M. Characterization of K-fluorrichterite based glass–ceramics for biomedical applications, in engineering materials. Sheffield: University of Sheffield; 2005. p. 211.
16. Bhakta S, Pattanayak DK, Takadama H, Kokubo T, Miller CA, Mirsaneh M, Reaney IM, Brook IM, van Noort R, Hatton PV. Prediction of osteoconductive activity of modified potassium fluorrichterite glass–ceramics by immersion in simulated body fluid. J Mater Sci. 2010;21:2979–88.
17. ISO 6872:1995, Dental Ceramics, International Organization for Standardisation, Geneva, Switzerland.
18. Devlin AJ, Hatton PV, Brook IM. Dependence of in vitro biocompatibility of ionomeric cements on ion release. J Mater Sci. 1998;9:737–41.
19. Wallace KE, Hill RG, Pembroke JT, Brown CJ, Hatton PV. Influence of sodium oxide content on bioactive glass properties. J Mater Sci. 1999;10:697–701.
20. Hurrell-Gillingham K, Reaney IM, Miller CA, Crawford A, Hatton PV. Devitrification of ionomer glass and its effect on the in vitro biocompatibility of glass ionomer cements. Biomaterials. 2003;24:3153–60.
21. Nakayama GR, Caton MC, Nova MP, Parandoosh Z. Assessment of the Alamar blue assay for cellular growth and viability in vitro. J Immunol Methods. 1997;204(2):205–8.
22. Stokes CW, Hand RJ, van Noort R. Investigation of the chemical solubility of mixed alkali fluorocanasite forming glasses. J Non-Cryst Solids. 2006;352:142–9.
23. Freeman CO, et al. Crystallization modifies osteoconductivity in an apatite–mullite glass–ceramic. J Mater Sci. 2003;14:985–90.